

## OPTIMISATION OF THE FORMATION AND DISTRIBUTION OF PROTOPORPHYRIN IX IN THE UROTHELIUM: AN IN VITRO APPROACH

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### ABSTRACT

### REFERENCE 1

**Purpose:** To optimize conditions for photodynamic detection (PDD) and photodynamic therapy (PDT) of bladder carcinoma, urothelial accumulation of protoporphyrin IX (PpIX) and conditions leading to cell photodestruction were studied.

**Materials and Methods:** Porcine and human bladder mucosae were superfused with derivatives of 5-aminolevulinic acid (ALA). PpIX accumulation and distribution across the mucosa was studied by microspectrofluorometry. Cell viability and structural integrity were assessed by using vital dyes and microscopy.

**Results:** ALA esters, especially hexyl-ALA, accelerated and regularized urothelial PpIX accumulation and allowed for necrosis upon illumination.

**Conclusions:** hexyl-ALA used at micromolar concentrations is the most efficient PpIX precursor for PDD and PDT.

**KEY WORDS:** aminolevulinic acid, photodynamic detection, photodynamic therapy, urinary bladder, cancer, in vitro

Urinary bladder tumors show an increasing incidence in man after the sixth decade. They consist mainly of superficial transitional carcinomas and are characterized by frequent recurrence and/or risk to progress toward invasive tumors.<sup>1</sup> This is linked to their frequent multifocal character and concomitant presence of high grade dysplasia (DYS) centers and/or carcinomas in situ (CIS).<sup>2</sup>

The treatment of superficial bladder tumors is based mainly on endoscopic resections combined with chemo- or immunotherapy by intravesical installation. While the use of BCG is likely to modify the recurrence profile of the illness, reduce the risk of progression and improve the survival,<sup>3</sup> the resistance to BCG of certain tumoral bladders and decrease of vesical compliance resulting from repetitive treatments remain therapeutic problems.

As an alternative, photodynamic therapy (PDT) aims at destroying malignant cells by inducing cytotoxic reactions which result from interaction of light with photosensitive endo- or exogenous compounds, often preferentially accumulating in the target tissues. This concept led to development of several oncological treatments, for example, in dermatology, otorhinolaryngology, gastrology, ophthalmology and gynaecology.

In urology, where the main indication for PDT is multi-recurrent superficial bladder cancer resistant to BCG treatment, PDT has received only marginal interest because the first generation photosensitizers did not localize with sufficient selectivity in neoplastic tissues and induced skin photosensitivity after systemic administration. Recently, interest in PDT of bladder cancers has been renewed by demonstration of the selectivity of protoporphyrin IX (PpIX) induced after instillation of 5-aminolevulinic acid (ALA). PpIX is an intermediate of the cycle of heme synthesis (fig. 1) and its intracellular content can be significantly increased when the regulatory step of the cycle is bypassed by exposing the tissue to a precursor, for instance 5-aminolevulinic acid (ALA).<sup>4</sup> In addition, PpIX accumulates at much higher con-

centrations in malignant than in normal cells due to the reduction of ferrochelatase and iron deficiency in tumors.<sup>5</sup>

The results obtained by PDT in skin tumors<sup>6</sup> suggested that a similar approach might be used in urology. While the diagnosis of CIS and DYS is difficult or impossible during cystoscopy using white light, fluorescence cystoscopy after intravesical administration of 3% ALA solution often allows us to detect and define with precision the limits of DYS and CIS.<sup>7,8</sup> The sensitivity and specificity of photodynamic detection (PDD) approach 80%. The preferential accumulation of PpIX in the transformed urothelium,<sup>9</sup> the intravesical tolerance of ALA solutions adjusted to physiological pH values, and the absence of systemic effects reinforce the interest of such an approach.

A complete destruction of a tumor by PDT critically depends on a sufficiently high concentration and homogeneous distribution of PpIX in the malignant cell layers.<sup>10</sup> Although relatively high ALA concentrations were instilled into the bladder for many hours, fluorescence microscopy showed a rather irregular distribution of PpIX within superficial tumors of the bladder.<sup>11</sup> Also, the conditions for reaching the threshold of phototoxicity in the urothelium are not exactly known. This is not surprising as a double charged molecule like ALA is not expected to penetrate with ease across cell membranes and interstitial spaces. More lipophilic derivatives of ALA are expected to be more favored from this point of view. After traversing the cellular membrane non-specific esterases will reduce such compounds to 5-ALA. Dimethylsulfoxide (DMSO) and desferrioxamine (DES) have been

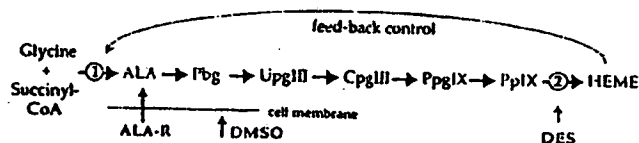


FIG. 1. Simplified scheme of heme biosynthesis and interventions used. ALA: 5-aminolevulinic acid; ALA-R: esters of ALA; Pbg: porphobilinogen; UpgIII: uroporphyrinogen III; CpgIII: coproporphyrinogen III; PpgIX: protoporphyrinogen IX; PpIX: protoporphyrin IX. 1: ALA synthase; 2: ferrochelatase + Fe<sup>++</sup>. DMSO: dimethylsulfoxide; DES: desferrioxamine. Gray arrows: inhibitory effects.

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## UROTHELIAL ACCUMULATION OF PROTOPORPHYRIN IX

and to enhance PpIX accumulation. DMSO increases the transmembrane passage of small molecules.<sup>12</sup> DES, chelates the intracellular iron and hence inhibits the ferrochelatase activity. Consequently, PpIX is not converted into hem (fig. 1) and accumulates in cells.<sup>6</sup>

Thus, to define standardized and optimal conditions for PDD and PDT, a systematic study of the penetration of ALA derivatives into cells, the kinetics of PpIX accumulation, intra-urothelial PpIX distribution and mechanisms of cell photodestruction is mandatory. As a first approach to this problem, we developed an experimental setup allowing us to answer some of these questions by using bladder mucosae explanted *in vitro*. Here we present the kinetics and tissue profiles of intracellular PpIX accumulation, and data about phototoxicity obtained in pig or human urothelium exposed to ALA, its esters, and ALA with DMSO or DES.

### MATERIALS AND METHODS

**Preparation of bladder mucosa.** The study required living urothelium obtained under controlled conditions. Porcine urothelium resembles human urothelium structurally<sup>13</sup> and can be obtained easily and reproducibly. Porcine bladders were excised from slaughtered animals. Pieces of human bladder wall were obtained from patients undergoing radical

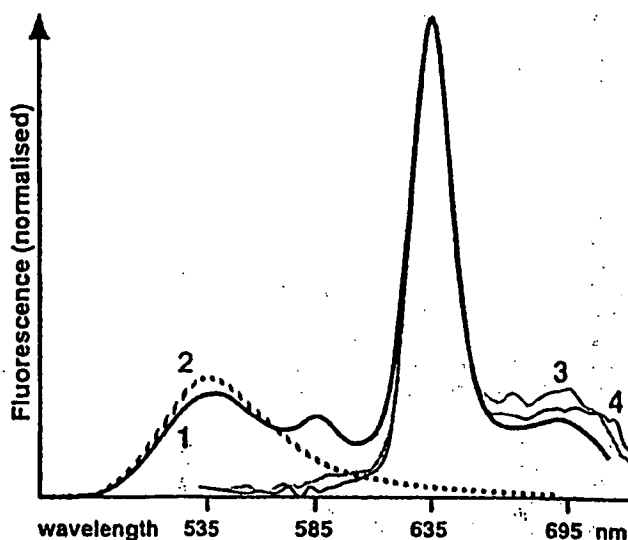


FIG. 3. Specificity of fluorescence signal. Four emission spectra as obtained from living mucosae and from frozen sections of urothelium. 1, living mucosa preincubated for 2 hours with ALA. 2, same mucosa after 10 minutes illumination. 3 and 4, urothelial sections incubated with ALA+DES and H-ALA, respectively. Spectra 1, 3 and 4 show identical peaks around 635 and 690 nm corresponding to PpIX accumulated in urothelium. In spectrum 2, PpIX signals disappeared leaving only tissue autofluorescence. Note that emission peak at 670 nm of curve 3 is due to photooxidation products of PpIX.

cystectomy for advanced carcinoma (3 males, 1 female; average age  $73 \pm 6$  years). Resected bladders were opened and *de visu* normal and flat areas were taken for experiments. All these manipulations took about 45 minutes. Normality was confirmed by histology. The protocols were approved by the state commissions controlling animal experiments and clinical research.

The tissues were stored at 4°C in Tyrode solution. The urothelium was microdissected from the bladder wall using fine scissors. The plane of cleavage passed as near as possible to the basal membrane so that thin sheets of urothelium with remnants of lamina propria connective tissue were obtained.

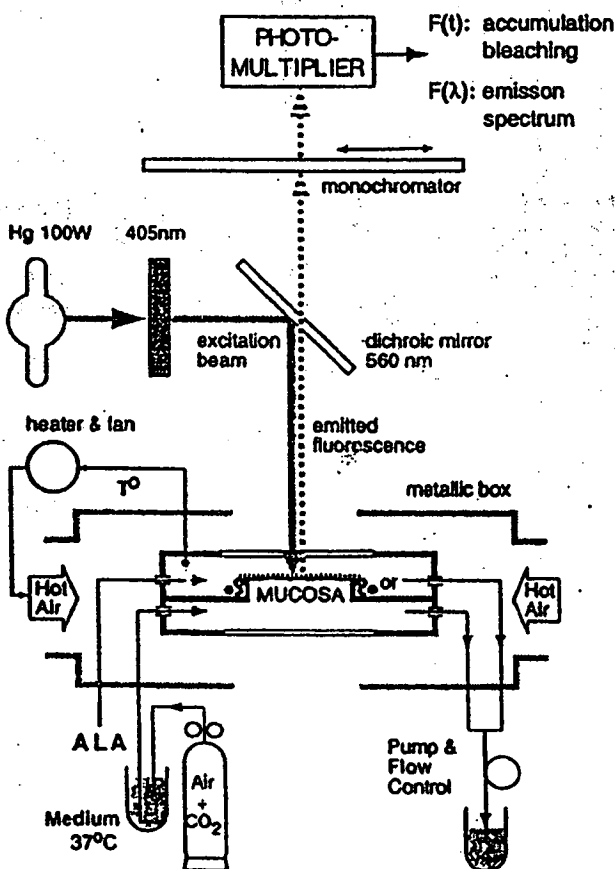


FIG. 2. Microspectrophotometry of protoporphyrin IX in bladder mucosa. Bladder mucosa is placed over and around circular rim of perspex plate and fixed with thin silicone O-ring (or). Preparation is mounted into transparent chamber and incubated in presence of ALA derivatives. Mucosa is periodically excited (100W mercury lamp, Eppendorf filter 405 nm (FWHM: 12 nm), 200 msec exposure,  $45 \pm 5 \mu\text{W}/0.05 \text{ mm}^2$ ) and fluorescence emitted by cells is recorded by using EMI 20 photomultiplier. Motorized monochromator (continuous interference filter Veril, Leitz) allows analysis of emission spectrum.

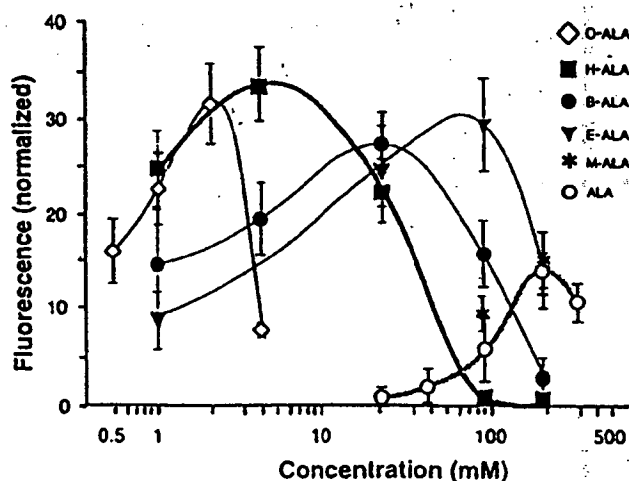


FIG. 4. PpIX kinetics for different precursors. O-ALA and H-ALA are most efficient precursors tested: at concentration 100 times lower than that of ALA, they induce 2 to 3 times higher PpIX accumulation. Determinations are made after 1 hour of incubation. Values are means  $\pm$  S.D. from 20 measurements (4 mucosae per condition, 5 measurements in each mucosa). Values for M-ALA, determined at 90 and 180 mM only, were  $9.4 \pm 1.8$  and  $15 \pm 3$  respectively.

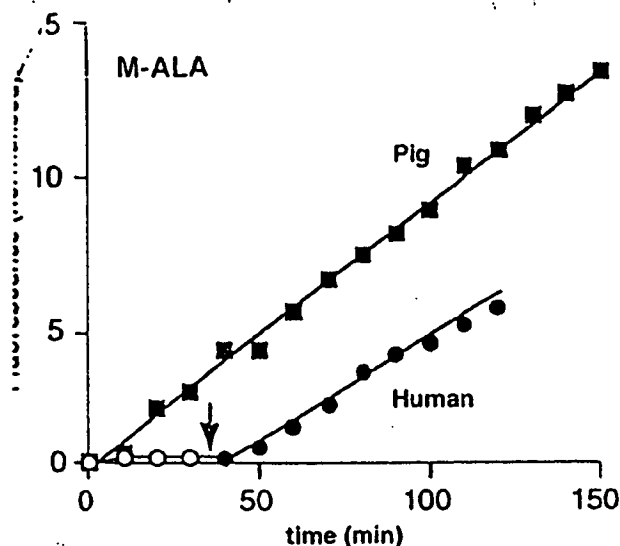


Fig. 5. Comparison of PpIX fluorescence in pig and human mucosa. During first 3 hours of incubation fluorescence increase is nearly equal in pig mucosa. Pig mucosa accumulates PpIX faster ( $F = 0.082 \text{ min}^{-1}$ ,  $r^2 = 0.99$ ) than human mucosa ( $F = 0.062 \text{ min}^{-1}$ ,  $r^2 = 0.98$ ). PpIX fluorescence is only observed when human mucosa is warmed up. M-ALA was administered at time 0. Arrow: warming of human mucosa; empty circles: human mucosa at 23°C; full symbols: mucosae at 36°C.

these were cut into  $7 \times 7 \text{ mm.}$  fragments which were mounted (urothelium up) in a transparent culture chamber designed for pithelia<sup>14</sup> as illustrated in fig. 1. The mucosa divided the chamber into superior and inferior compartments (diameter 20 mm., height 3 mm.) and the area exposed to exchanges was  $0.125 \text{ cm.}^2$ . The chamber was fixed onto the plate of an epi-illumination microscope (Leitz Orthoplan) and thermostabilised at  $36 \pm 0.5^\circ\text{C}$ . The inferior compartment was continuously perfused by oxygenated Tyrode solution. Solutions of ALA derivatives were injected as a single dose into the superior compartment.

**Media.** The Tyrode solution contained (in mmol/L):  $143.0 \text{ Na}^+$ ,  $2.0 \text{ K}^+$ ,  $0.8 \text{ Mg}^{++}$ ,  $1.4 \text{ Ca}^{++}$ ,  $122 \text{ Cl}^-$ ,  $20.0 \text{ HCO}_3^-$ ,  $3 \text{ I}_2\text{PO}_4^-$ ,  $1.2 \text{ SO}_4^{--}$ , 8 glucose (osmolarity 290 mOsm/L) and was saturated with air enriched with 5%  $\text{CO}_2$  (pH 7.5). ALA and some of its derivatives were dissolved in phosphate buffer saline at  $4^\circ\text{C}$ , and the pH was adjusted to  $5.2 \pm 0.5$ . Dimethylsulfoxide (DMSO) ( $17.5 \mu\text{M}$ ) and desferrioxamine mesylate (DES) ( $15 \mu\text{M}$ ) were added to some solutions. All solutions were colorless. They were stored on ice and used within one hour.

ALA was from Merck (Dietikon, Switzerland), methyl-ester

(M-ALA) and DES were from Fluka (Buchs, Switzerland). Ethyl-(E-ALA), butyl-(B-ALA) hexyl-ester (H-ALA) and octyl-ester (O-ALA) were synthesized.<sup>15</sup> Their purity was superior to 95%.

**Spectrofluorometry.** The kinetics of urothelial PpIX accumulation with respect to precursor concentration and to time of administration were characterized as follows (fig. 1). The urothelium, incubated with a given precursor, was excited by violet light ( $405 \text{ nm}$ ,  $45 \pm 5 \mu\text{W}/0.05 \text{ mm.}^2$ , 200 ms) each 10 minutes or each hour and the fluorescence emitted by the cells, which is taken to be proportional to the cell PpIX concentration, was passed through a low pass filter ( $>610 \text{ nm}$ ) and recorded by a photomultiplier. The specificity of the fluorescence signal was systematically checked by analyzing the emission spectra.

The spatial distribution of PpIX across the mucosa was determined at selected time intervals in serial  $25 \mu\text{m.}$  thick frozen sections. To avoid strong photobleaching due to light exposure, the samples were prepared in the dark. The profiles of PpIX fluorescence within the mucosa were determined by scanning the fluorescence signal across the section.

**Cell viability.** At the end of experiments, the urothelium was exposed to acridine orange (dissolved in Tyrode 1:10000) which stains nuclei of living cells only. The proportion of labeled nuclei was evaluated by fluorescence microscopy (excitation at  $405 \text{ nm}$ , emission  $> 560 \text{ nm}$ ). In some cases, the time-course of PpIX photodestruction (photobleaching) was determined and the consequent phototoxicity effects on urothelial cell were studied by using electron microscopy. Two hours after the exposure to light, the mucosae were fixed in paraformaldehyde/glutaraldehyde and embedded in Epon. Sections of  $700 \text{ \AA}$  were analyzed by transmission microscopy (Zeiss, Germany). Some mucosae were dehydrated and dried (CPD 030 critical point dryer, Balzers, Liechtenstein), coated with  $300 \text{ nm}$  gold (S150 sputter coater, Edwards, Ziviy, Basle) and studied by using scanning electron microscope (JEOL, Tokyo).

**Statistical analysis.** Supposing that the photobleaching of endogenous chromophores is small, fluorescence values were normalized, that is, corrected for the tissue autofluorescence ( $I_n(t) = (I_n(t) - AF)/AF$ ). The data are presented as arithmetical means and standard deviations. A paired bilateral  $t$  test was used to compare the results and values of  $p \leq 0.05$  were considered as significant.

## RESULTS

**Nature of the fluorescence signal.** The spectral analysis of the light emitted by the mucosa shows that, in the absence of PpIX precursors, the tissue emits weak autofluorescence giving a very small signal in the red domain ( $>610 \text{ nm}$ ). This signal serves to normalize the specific PpIX fluorescence. The latter appears in presence of PpIX precursors as one major

Pp IX accumulation in urothelium in response to ALA derivatives

Precursor	Hours of Administration						
	1	2	3	4	5	6	7
ALA	$17 \pm 7$	$31 \pm 7$	$41 \pm 6$	$54 \pm 9$	$64 \pm 7$	$67 \pm 10$	$65 \pm 12$
ALA+DMSO	$14 \pm 3$	$36 \pm 8$	$57 \pm 17$	$75 \pm 17$	$90 \pm 18$	$90 \pm 18$	$87 \pm 31$
M-ALA	$15 \pm 3$	$35 \pm 5$	$53 \pm 5$	$69 \pm 9$	$82 \pm 13$	$94 \pm 14$	$91 \pm 20$
M-ALA+DMSO	$18 \pm 3$	$42 \pm 5$	$63 \pm 9$	$79 \pm 11$	$97 \pm 14$	$115 \pm 13$	$114 \pm 17$
ALA+DES	$21 \pm 2$	$48 \pm 11$	$81 \pm 13$	$117 \pm 14$	$157 \pm 36$	$193 \pm 50$	$256 \pm 27$
B-ALA	$27 \pm 3$	$61 \pm 7$	$100 \pm 14$	$134 \pm 28$	$163 \pm 28$	$156 \pm 27$	$144 \pm 25$
H-ALA	$34 \pm 4$	$67 \pm 6$	$105 \pm 12$	$134 \pm 20$	$167 \pm 27$	$175 \pm 26$	$189 \pm 37$
H-ALA+DES	$32 \pm 3$	$62 \pm 5$	$98 \pm 4$	$126 \pm 10$	$153 \pm 7$	$172 \pm 8$	$170 \pm 16$

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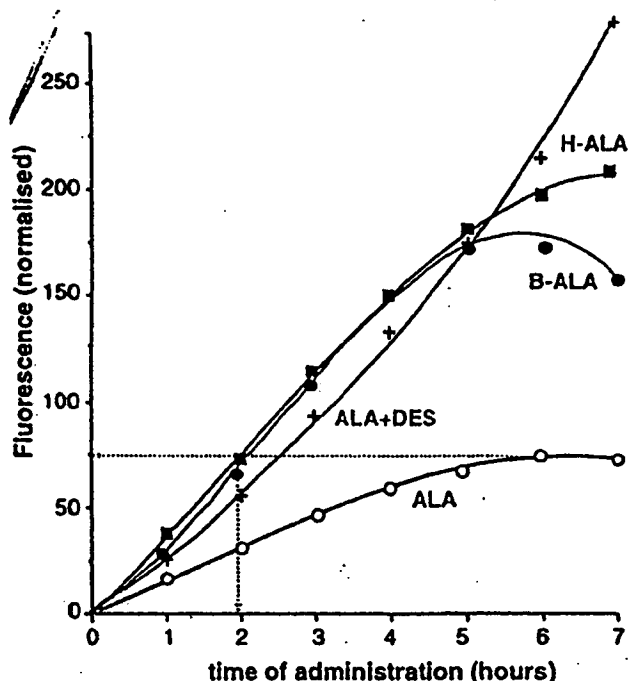


FIG. 6. PpIX accumulation with ALA, ALA+DES, B-ALA and H-ALA. H-ALA, although administered at much lower, and hence less toxic concentration, can considerably shorten time of administration (dotted lines), allowing both rapid and efficient PDT. (ALA and ALA+DES: 700 mOsm; H-ALA: 290 mOsm). Values are means extracted from table.

(635 nm) and one minor (690 nm) peak (fig. 3). No differences were observed between the emission spectra shape of pure ALA- and ALA-esters-induced PpIX. The PpIX peaks disappear after exposure of the mucosa to light.

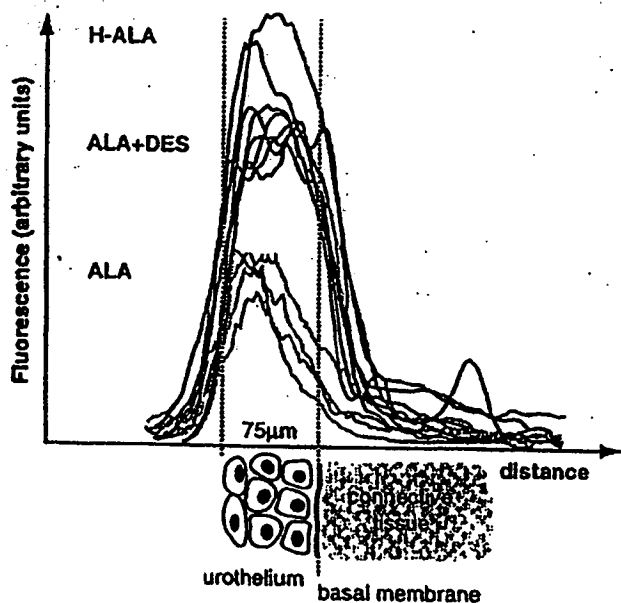


FIG. 7. Fluorescence intensity as measured across bladder mucosa. H-ALA allows highest and most homogeneously distributed PpIX accumulation in urothelium. Data from 12 fluorescence scans across mucosal sections were corrected to mean urothelial thickness. Scanning speed 100 mm/sec.; excitation at 405 nm.; width of illuminated slit 30 µm.; emission at 610 nm).

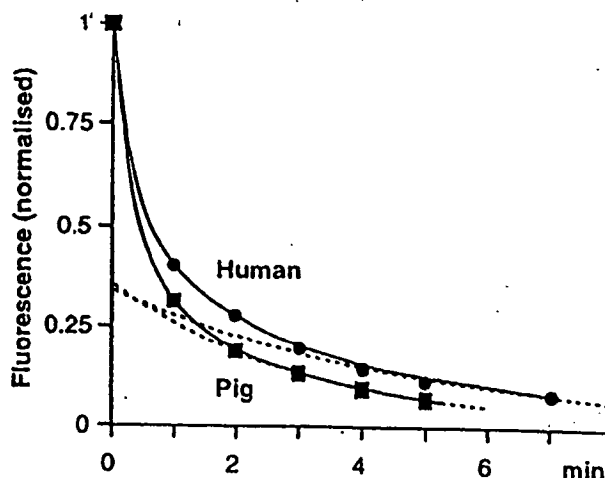


FIG. 8. Photosensitivity of fluorescence signal. Time-course of photobleaching of PpIX upon continuous illumination (405 nm, 4.75 J/cm<sup>2</sup>) of mucosa. Photobleaching appears to show fast and slow phases. Residual signal corresponds to autofluorescence.

**Effect of PpIX precursor concentration.** Fig. 4 illustrates the results obtained for ALA and its esters after 1 hour of incubation. All precursors show similar kinetics: with increasing concentration, the fluorescence intensity increases, reaches a maximum and then decreases sharply. As compared with ALA, the applied concentrations of E-ALA, B-ALA, H-ALA and O-ALA are respectively 2, 10, 45 and 90 times lower, but nevertheless result in 2 to 2.5 times higher fluorescence.

The reduction of fluorescence at high concentrations may indicate significant cytotoxicity of ALA esters which resulted in peeling of the mucosa (as with ALA at 180 mM), or even immediate cellular lysis (as with H- and B-ALA at 180 mM) (not shown). Due to its higher lipophilicity, precipitation of O-ALA in aqueous solutions at high concentrations may reduce the total drug content.

**Accumulation of PpIX in the mucosa.** Both the pig and human mucosae exposed to precursors accumulate PpIX, after warming to 37°C, that is, upon metabolic activation. This is illustrated in fig. 5 which shows an example of human mucosa with an accumulation ratio comparable to that of pig mucosa. However, on the average, the human mucosae (n = 4, 3 males, 1 female, mean age 52 years) accumulated PpIX 3.6 times less than the pig mucosae.

The table shows the results obtained in the pig mucosae with ALA derivatives used at their respective optimal concentrations. In all cases, the fluorescence increased nearly linearly up to four hours and saturated between the 6th and 7th hours. With ALA+DES, the fluorescence continued to increase exponentially up to 7 hours. ALA was the least efficient of the tested precursors. The other substances induced a significantly faster and greater (1.3 to 3 times) increase of PpIX fluorescence. H-ALA and ALA+DES were the most efficient, but H-ALA and B-ALA were shown to reach the highest fluorescence at the shortest administration times (table, fig. 6). No significant difference in PpIX formation was observed between H-ALA and B-ALA used at their optimal concentrations. This indicates that the biosynthetic pathway of heme was saturated by the more lipophilic esters, while PpIX production induced by pure ALA never reached sufficiently high intracellular drug contents.

Iso-osmolar replacement of sodium in the Tyrode solution by cholin did not modify the accumulation kinetics of PpIX (n t shown) indicating that the penetration of ALA derivatives into the cell does not involve a sodium-dependent co-transport.

At the end of each experiment (24 mucosae, 7 hours of

incubation, 8 precursors at their optimum concentration), on labeling with acridine orange, all preparations showed cells with bright nuclear fluorescence, indicating that the epithelium remained alive.

**Distribution of PpIX across the mucosa.** The fluorescence profiles across the mucosae were recorded after 2 hours of incubation with ALA, H-ALA and ALA+DES. As the thickness of urothelium varied (from 66 to 88  $\mu\text{m}$ ), the results are presented after a homothetic translation to 75  $\mu\text{m}$ . As shown in fig. 7, PpIX fluorescence induced by the 3 precursors is limited essentially to the urothelial cells. With ALA, the fluorescence is limited mostly to the superficial cells while with ALA+DES and especially H-ALA, the fluorescence is about twice as high and distributed in all urothelial layers.

**Phototoxicity.** If the mucosa accumulating PpIX is exposed to continuous violet illumination for 10 minutes, the urothelial fluorescence decays. Supposing an exponential decay with time, the two rate constants are of about 30 seconds and 3 minutes (fig. 8). This time dependence of the fluorescence signal may be due to more stable photoporphyrins formed by photodegradation of PpIX. After 10 minutes of illumination, the

specific fluorescence is no longer detectable which indicates that most fluorescing porphyrins were destroyed.

When the mucosae illuminated for 10 minutes were incubated for 2 additional hours, the cells that had been exposed to light died. This was documented by electron microscopy (fig. 9) which revealed damaged mitochondria, marginalisation of nuclear chromatin, vacuolised cytosol and fenestration of the plasma membrane. The superficial cells were rounded and lost contact with each other. In mucosae preincubated for shorter times (for example, ALA, 2 hours), the necrotic changes were found mostly in the superficial cells. In mucosae preincubated for longer times (for example, ALA, 6 hours) the urothelial necrosis was complete while the underlying connective tissue was not damaged. The necrosis induced by violet light was confined to the illuminated area and was surrounded by normal cells (fig. 9).

#### DISCUSSION

The use of bladder mucosa explanted into a superfusion chamber is a powerful tool which, unlike cell cultures, per-

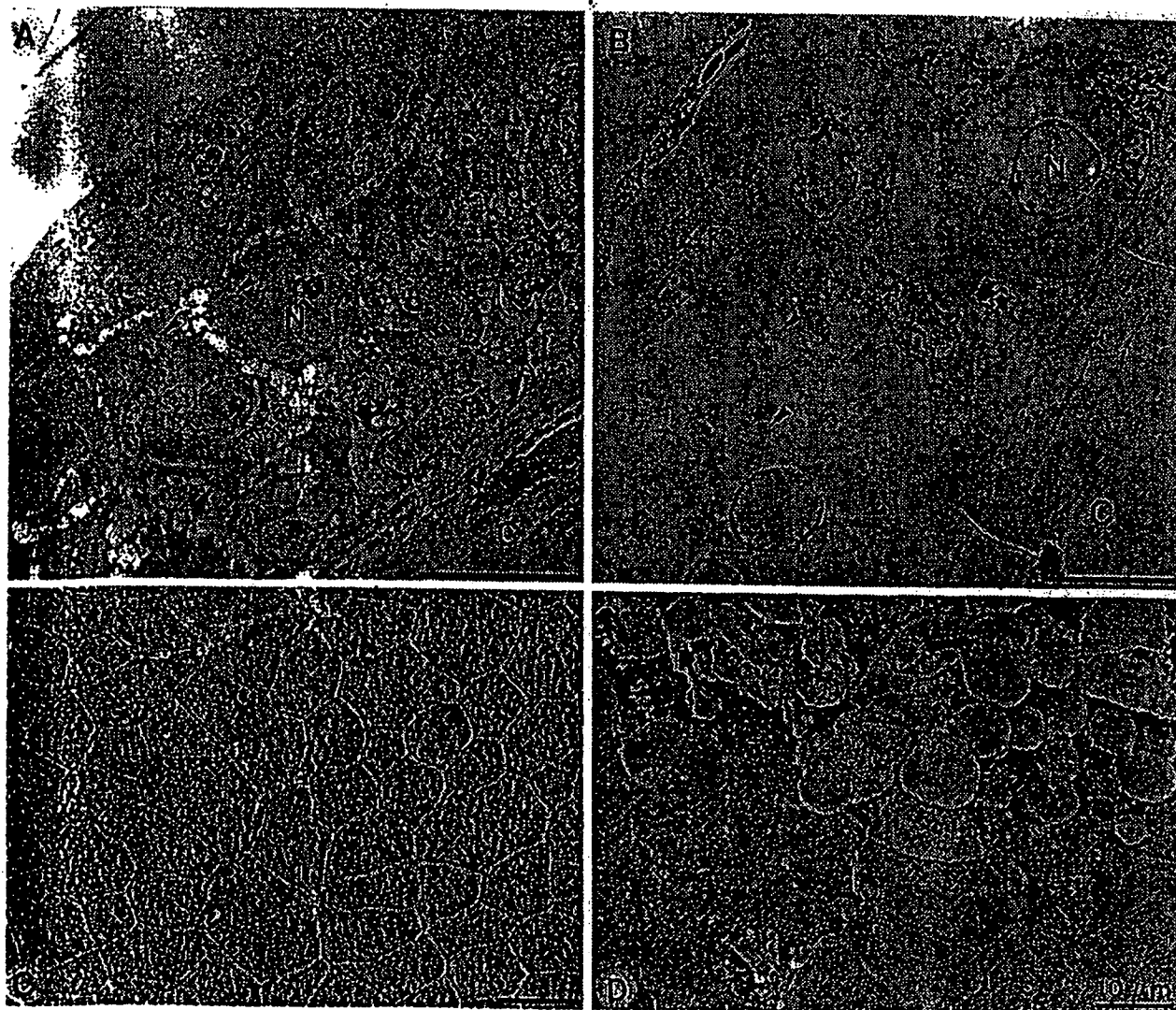


FIG. 9. Urothelial necrosis induced in bladder mucosae by exposure to light. Transmission (A, B) and scanning (C, D) electron micrographs of mucosae incubated for 6 hours with ALA. A, C, control mucosae with normal intra- and intercellular structure. B, D, mucosae exposed to light (45 nm, 4.75 J/cm<sup>2</sup>, 10 minutes) and incubated for 2 additional hours, showing marginalized chromatin, swollen mitochondria, vacuolized cytoplasm, fenestrated plasma membrane and lost intercellular contacts. Arrows: mitochondria, N: nuclei, C: connective tissue. Bars: 10  $\mu\text{m}$ .

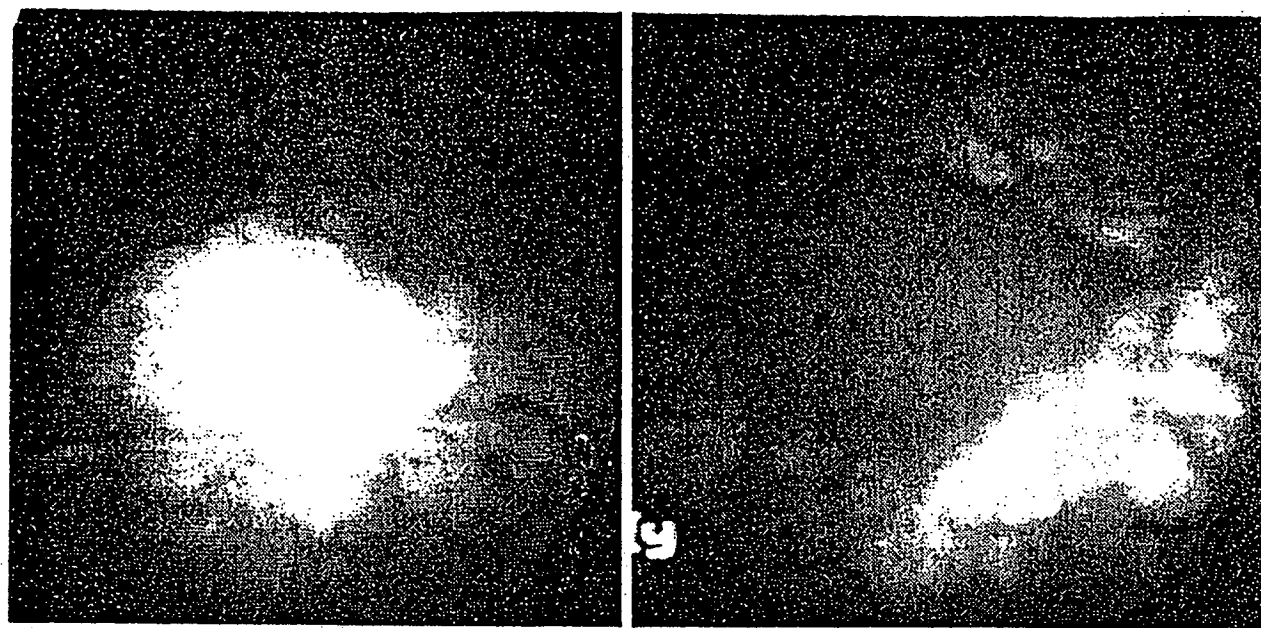


FIG. 10. Endoscopic view of human bladder papilloma. Left: image in white light. Dimension of tumor is 4 × 8 mm. Right: image in near-violet light after instillation of hexyl-ALA (8 mM) for 2 hours. Highly fluorescent tumoral cells (red) appearing on background of non-fluorescent (green) normal tissue. Histological diagnosis: pTaG1. (With permission.<sup>24</sup>)

its the study of epithelia with intact architecture and functional polarity. This is especially valuable for evaluation of penetration of substances into the normal as well as pathological epithelia. Although the blood circulation is eliminated in this preparation, the renewal of submucosal medium serves the homeostasis and survival of urothelial cells.

Urothelium of pig and human origins has a very similar structure although surface proteoglycans are not identical and mucous cells are absent in the normal human bladder.<sup>13</sup> In our hands, both mucosae also show similar accumulation kinetics although the final fluorescence intensity in the human case is lower. This reflects possibly decreased cell viability due to unavoidable and long (about 2 hours) hypoxia resulting from early vascular ligation during the bladder preparations.

The synthesis of supplemental PpIX must be preceded by penetration of the precursor across the plasma membrane. Three results strongly suggest that the precursors penetrate into the cells by simple diffusion:<sup>1</sup> esters with the longer lipophilic moiety (hence more lipophilic) penetrate faster and to a greater extent,<sup>2</sup> similar kinetics of PpIX formation for H-ALA (4 mM) and B-ALA (20 mM) as well as comparable fluorescence levels under optimized concentrations suggesting a passive concentration gradient driven uptake as predicted by simple diffusion laws,<sup>3</sup> and absence of sodium in the solutions does not decrease the PpIX accumulation as would be expected for a sodium-linked co-transport frequently operating for amino-acid cell transport. This is in agreement with results obtained in cell cultures.<sup>16</sup>

Once inside the cell, the esters of the ALA are hydrolyzed by non-specific cell esterases and free ALA appears in the cytoplasm.<sup>16</sup> The cell fluorescence will, however, increase only upon metabolic activation in the mitochondria, which confirms that PpIX synthesis is an energy-dependent process (Fig. 5).

Whatever the precursor used, the time profiles of accumulation are similar: with increased concentration, the synthesis of PpIX increases to a maximum and then decreases to zero. Similar results were obtained in cell cultures.<sup>16</sup> In our case, the final decrease is accompanied by a loss of cell integrity and presence of free cells floating in the superfusate.

This might be due to the hypertonicity of the solutions<sup>17</sup> (Fig. 4) and/or to the toxicity of ALA itself. Indeed, the cells also peel off in presence of diluted but highly penetrant precursors such as H-ALA where the resulting high cytoplasmic ALA concentration might favor the production of oxygen reactive radicals<sup>18</sup> and subsequent cell injury.

PpIX fluorescence increases essentially linearly up to 4 hours and then attains a plateau value or even slightly decreases. Similar results were found in cell cultures from tumoral bladders and in rat urothelium *in vivo*.<sup>19, 21</sup> It is possible that this plateau reflects not only the balance between PpIX synthesis and PpIX utilization, which should happen with all precursors, but also the penetration of precursors into deeper lying cells, which should increase with liposolubility of the ALA esters, and appears to be shown by the fluorescence profiles. The observed dependence of decreasing optimal concentration with increasing chain length of the ALA derivatives has been confirmed in cell cultures.<sup>16</sup> O-ALA at higher concentrations shows a tendency to precipitate at physiological pH values which limits its clinical use.

Clearly, DMSO, which is supposed to increase the membrane permeability, potentiates the PpIX accumulation under our conditions much less than the esterification of ALA. DES, a selective iron chelator, when combined with ALA, inhibited the conversion of PpIX to heme and thus potentiated PpIX accumulation to higher levels and before a plateau was reached. This suggests that the synthesis of PpIX in itself was not slowed down in any of the conditions used. Surprisingly, DES showed no potentiation when combined with H-ALA. The explanation of this result awaits new experiments.

The photobleaching curves show a fast and a slow component, which might result from the bleaching of fluorescent photooxidation products, originating in different intracellular compartments.<sup>22</sup> A mixture of isomeric chlorins resulting from the first photodegradation process of PpIX has been shown to be about 10 times more stable to photooxidation than PpIX.<sup>23</sup> Their appearance may be seen from the fluorescence emission peak at 670 nm (Fig. 3, curve 3). Our results show that 10 minutes exposure to light is sufficient to induce cell necrosis in all layers of a normal urothelium. In



no records of photobleaching could help to dose the radiation energy necessary to destroy the tumoral tissue.

# CONCLUSIONS

The in vitro preparation of bladder mucosa developed in this work has brought additional valuable information on the kinetics of accumulation and destruction of photosensitive molecules used in the PDD and PDT of urothelial carcinoma. In the case of PpIX, H-ALA seems to be a good compromise between lipophilicity, solubility and performance with respect to high PpIX formation and low precursor concentration. In comparison with ALA, it increases and accelerates PpIX synthesis, penetrates into all epithelial cell layers, and is efficient at low concentrations. At these low concentrations it preserves urothelial viability and allows effective photodestruction. The optimal time necessary for PDD and PDT at the conditions applied is shortened from near 6 to 1 hours. As shown in fig. 10, under these conditions applied to humans, PDD results confirm the predictions and show effective accumulation of PpIX in urothelial carcinoma.

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